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Absence of Serological Evidence for Foamy Virus Infection in Patients With Amyotrophic Lateral Sclerosis

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Foamy virus (FV) infection has been implicated in the pathogenesis of sporadic motor neuron disease (MND) by means of serological assays. To confirm these results we tested serum and cerebrospinal fluid (CSF) samples from 23 cases of clinically verified non-familial MND and 11 cases of suspected non-familial MND for the presence of FV infection as determined by Western blot (WB) and indirect immunofluorescence assay (IFA). Using the same tests we also screened sera from 87 healthy chimpanzees for the presence of FV antibodies. None of the human samples in question tested positive. However, the testing revealed that 84 of 87 chimpanzees (96.6%) were seropositive for FV, indicating that combined WB and IFA are suitable methods for the serodiagnosis of FV infection. Given these results an association of FV infection and sporadic MND is highly improbable. Furthermore a suggested therapeutic trial with anti-retroviral drugs appears unjustified.

KEY WORDS: human foamy virus, amyotrophic lateral sclerosis, motor neuron disease, serology

INTRODUCTION

Motor neuron disease (MND) is a progressive disorder in which degeneration of upper and lower motor neurons in the absence of an inflammatory reaction leads to progressive weakness of bulbar, limb, thoracic, and abdominal muscles. From 90 to 95% of cases are sporadic, 5–10% are familial. The yearly incidence of MND is 1–2 per 100,000 in most parts of the world [Leigh and Ray-Chaudhuri, 1994]. The aetiology of non-familial MND is unknown. Several different viruses, in particular enteroviruses, have been implicated in the aetiology of MND in the past [Rowland, 1984, 1994; Jubelt, 1992]. However, the issue is controversal and attempts to transmit amyotrophic lateral sclerosis (ALS) to non-human primates could not confirm an infectious agent as the cause of ALS [Rowland, 1984; Jubelt, 1992].

Two human retroviruses are associated with neurological disorders. Human immunodeficiency virus (HIV) is the causative agent of the AIDS dementia complex (ADC) [Wigdahl and Kunsch, 1989] while Human T-cell lymphotropic virus type I (HTLV-I) is linked to a distinct condition known as tropical spastic paraparesis (TSP) and to HTLV associated myelopathy (HAM) [Iwasaki, 1993]. Human foamy virus (HFV) is a representative of the spumavirus subgroup of retroviruses [Rethwilm, 1995]. HFV is a unique human isolate and is closely related to FVs from chimpanzees (SFVcpz) [Herchenröder et al., 1994; Schweizer and Neumann-Haefelin, 1995]. FVs are common in non-human primates, felines, and bovines [Hooks and Gibbs, 1975]. Accidental transmissions have clearly demonstrated that humans are susceptible to primate FV [Schweizer et al., 1994 and 1995]. In their natural hosts FVs give rise to persistent, apparently asymptomatic infections in the presence of high titers of antibodies [Hooks and Gibbs, 1975]. As revealed by virus isolation and detection of FV DNA the CNS is often involved in these infections [Hooks and Gibbs, 1975; Neumann-Haefelin et al., 1993].

Studies on the potential association of HFV with neurological diseases were initiated following the discovery that mice transgenic for HFV developed a severe encephalopathy and myopathy in the absence of inflammatory indications [Bothe et al., 1991; Aguzzi et al., 1993]. In addition, a condition which somehow resembles MND has been found in transgenic mice containing the env gene of a neurotropic murine retrovirus [Kay et al., 1993], suggesting a possible involvement of a retrovirus in the pathogenesis of MND [Rowland, 1991; Jubelt, 1992]. Recently, it has been reported that HFV antibodies are prevalent in sera of sporadic MND patients and

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an aetiological linkage between virus infection and disease has been suggested [Westarp et al., 1992].

Since knowledge of the causative agent of MND would have a great impact on the understanding of its pathogenesis and would offer therapeutic and prophylactic approaches to combat this fatal disease, we aimed to confirm the finding of anti-HFV antibodies in the serum of patients with sporadic MND.

MATERIALS AND METHODS

Materials

Twenty-two serum and corresponding CSF samples were taken from patients at the Neurologische Universitätsklinik, Tübingen. According to the criteria proposed for diagnosis of MND in clinical trials 15 patients were classified as having definite ALS, three as having probable ALS, three as having possible ALS, and one as having suspected ALS [Swash and Leigh, 1992]. An additional 12 serum and corresponding CSF samples from eight patients with clinically definite ALS, three patients with probable ALS, and one patient with suspected ALS were obtained from the Neurologische Universitätsklinik, Würzburg. All ALS cases in this study were non-familial. The mean age of the 34 patients at the time of diagnosis was 64 years (range 37 to 79 years), 18 of whom were males. Chimpanzee sera were collected from the colony of the Biomedical Primate Research Center, Rijswijk. Rhesus monkey sera and CSF samples were kindly provided by S. Sopper and S. Hemm, Institut für Virologie und Immunbiologie, Würzburg.

Cells and Viruses

Baby hamster kidney cells (BHK-21) were maintained in MEM supplemented with 5% foetal bovine serum and antibiotics. Cells infected with the HFV isolate of Achong et al. [1971] or with simian foamy virus (SFV) types 1 and 2 [Johnston, 1971; Hooks and Gibbs, 1975] were harvested as recently described [Hahn et al., 1994]. Extra-cellular HFV was purified from concentrated cell-free supernatant by sucrose gradient centrifugation as described earlier [Rethwilm et al., 1987].

Western Blot Analysis

Lysates from infected cells were resolved in SDS containing 11.5% polyacrylamide gels in a tricine buffer system [Schägger and von Jagow, 1987], and semi-dry blotted onto nitrocellulose membrane (Schleicher & Schüll, Dassel, Germany). The amount of protein loaded onto the gels was adjusted to give a clear positive signal with human or non-human primate positive control sera at a 1:1,600 dilution. Blots were blocked, reacted with test sera (diluted 1:100) or CSF (diluted 1:10), and developed as described previously [Hahn et al., 1994]. In addition, eight serum and CSF samples were additionally analyzed using gradient purified virus. In this case 1 μg of purified virus was applied per lane.

Indirect Immunofluorescence

BHK-21 cells and HFV infected BHK-21 cells were seeded into LabTek tissue culture chambers (Miles Laboratories, Naperville, Ill.), fixed after 48 hr incubation in cold methanol and indirect immunofluorescence was performed essentially as described previously [Schweizer et al., 1995] using fluorescein coupled second antibody (Dako). Human test sera were analyzed at two dilutions (1:10 and 1:40), while positive control sera and chimpanzee sera were diluted 1:40 and 1:160 in PBS containing 1% BSA.

RESULTS

The laboratory diagnosis of FV infection of humans has been hampered by the lack of clear criteria for the interpretation of serological assays. This problem was partially due to the low number of positive human samples which could serve as a reference for the assays and partially due to the limited knowledge of FV proteins. This has led to controversial results on the seroprevalence of FV antibodies in humans in the past [Achong and Epstein, 1978; Brown et al., 1978; Muller et al., 1980; Loh et al., 1980].

Criteria for an FV serodiagnosis have been established only recently [Schweizer et al., 1994, 1995]. This was done by using apes, monkeys, and a few well documented cases of accidental human infections from which FV could be either isolated or unequivocally demonstrated by PCR as positive references. Current criteria include demonstrable antibodies against the p70/74^{gag} precursor molecules in Western blot (WB), together with positive immunofluorescence assay (IFA) as judged by antibodies against nuclear antigen in infected cells [Schweizer et al., 1995; Schliephake and Rethwilm, 1994]. Additional criteria include the presence of antibodies against the non-structural p60 Bet-protein, which have been reported in 70% of Gag reactive chimpanzees [Hahn et al., 1994]. While Gag and Bet antibodies are readily detectable in WB, antibodies against the Env proteins (gp130, gp80, and gp47) are preferentially detected by radioimmunoprecipitation assay (RIPA) [Netzer et al., 1990; Giron et al., 1993; Hahn et al., 1994].

Applying the new criteria for serologic diagnosis we screened serum samples from 87 chimpanzees at the BPRC for FV antibodies by WB and IFA. It has been shown previously that chimpanzee FVs and the single human isolate are antigenetically indistinguishable and thus interchangeable as the source of virus antigen in these assays [Nemo et al., 1978; Herchenröder et al., 1994; Hahn et al., 1994]. As shown in Figure 1, antibodies directed against the HFV Gag and Bet proteins were easily detected in the chimpanzee serum samples by WB. Of the 87 sera tested, 84 (96.6%) had antibodies against the p70/74^{gag} precursor proteins. These sera also reacted positive in indirect immunofluorescence (data not shown). In addition, 72 (85.7%) of the positive reacting sera recognized the p60 Bet protein, corroborating previous findings indicating that Bet is among the immunodominant proteins in infected hosts [Hahn et al., 1994]. Following demonstration that our assay was suitable for the detection FV infected primates, we screened human MND samples for evidence of FV antibodies. Consistent data from IFA and WB assays did not reveal a single

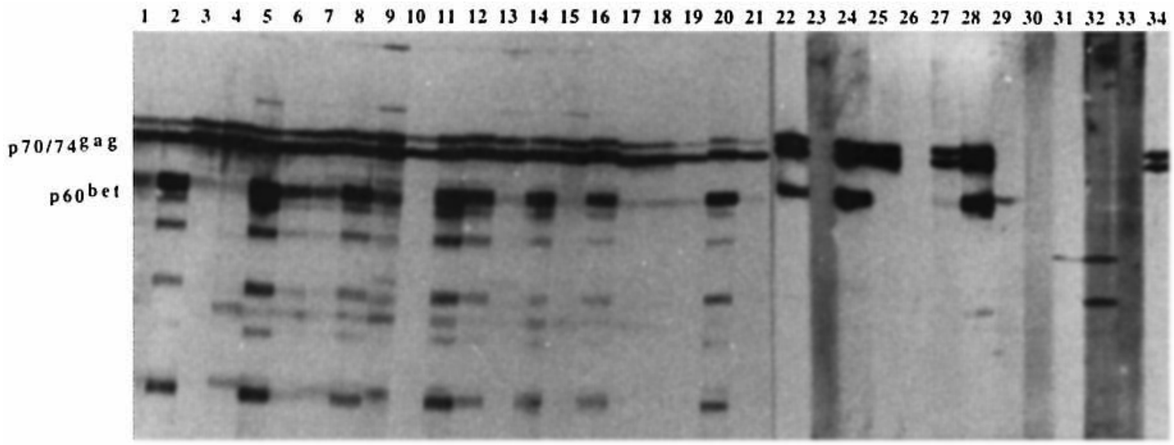

Fig. 1. WB analysis of 26 chimpanzee (**1-21** and **24-28**) and five MND sera (**29–33**), human positive and negative control sera (**22** and **23**, respectively), and rabbit α-HFV Gag serum (**34**) with antigen from HFV infected BHK-21 cells. Test sera were diluted 1:100 while control sera were diluted 1:400. Most Gag reactive sera also recognized the Bet protein, and only one chimpanzee (**26**) was found to be seronegative. None of the MND sera specifically detected any HFV antigens.

positive case. However, occasionally MND sera recognized unspecific bands in WB as shown in Figure 1. Furthermore, no HFV antibodies were detected when eight MND specimens were tested in WB strips which had been coated with gradient purified virus (data not shown).

Since antiviral CSF antibodies are often detected in retrovirus infections of the CNS [Resnick et al., 1985; Dörries et al., 1989], we then investigated the possibility that MND patients might preferentially have antibodies against FV in their CSF. To evaluate the sensitivity of the test system for the detection of FV specific CSF antibodies, we titered plasma and CSF from an FV infected Rhesus monkey on WB strips coated with an antigen mixture of the two macaque isolates SFV-1 and -2. The intensity of the gag specific bands (p70/74) at a plasma dilution of 1:3,200 was found to be similar to the CSF dilution of 1:10 (data not shown). Taking the corresponding plasma and CSF IgG concentrations (5.02 mg/dl and 0.0067 mg/dl, respectively) the intrathecal synthesis of virus-specific IgG was determined by the ratio: antiviral titer (CSF) × IgG (plasma) divided by antiviral titer (plasma) × IgG (CSF) [Ukkonen et al., 1981]. The value of 2.3 is borderline with respect to the indication of an intrathecal synthesis of FV-specific antibodies in this monkey [Sopper et al., 1993]. The result shows that FV antibodies may be detected in CSF samples of infected animals. We therefore tested the MND samples at the highest CSF concentration (1:10 dilution). However, none specifically reacted with HFV antigen in WB assay.

DISCUSSION

While there are clear genetic data on the pathogenesis of some hereditary forms of MND, the cause of sporadic MND remains unknown [Rowland, 1995]. In recent reports an association of sporadic MND with serologic markers of FV infection has been suggested [Westarp et al., 1992, 1993a, 1993b]. To confirm these studies we investigated sera and CSF samples from non-familial MND cases originating from roughly the same geographic area for FV antibodies. In the initial report Westarp et al. [1992] investigated sera from 308 individuals by ELISA using recombinant, subgenomic HFV Gag and Env antigen. Fifty-eight (18.8%) of the samples were found to be ELISA reactive and 29 (9.4%) were reported to detect HFV Gag antigen in WB, however, no figures for these WB positive cases were presented. The study included samples from 23 sporadic MND cases. Of these, 11 (47.8%) were reported to be ELISA positive and seven (30.5%) were reported to be WB positive. Extrapolating from these data, one would expect at least seven WB positive samples among the 23 sera from definite MND cases we tested. However, we were unable to identify a single positive case. A lack of sensitivity of our assays is unlikely since we identified by the same methods a high percentage of chimpanzees infected with FV.

Using similar tests as Westarp et al. [1992], Mahnke et al. [1992] reported FV antibodies in 6% of African sera and 2.7% of German patients' sera which has led to the conclusion that FV are possibly present worldwide in the human population [Flügel, 1993]. This view has been challenged recently by Schweizer et al. [1995], who investigated similar "at risk" populations and did not find any evidence of naturally occurring human FV infections. A likely explanation for the conflicting results between the studies of Maknke et al. [1992] and Westarp et al. [1992] on the one hand, and Schweizer et al. [1995] and this report on the other, is a lack of positive controls leading to an over-interpretation of false positive ELISA results in the former. Since human FV infections have so far been solely identified as apparently benign and

very rare zoonoses in laboratory and monkey house personnel, it is evident that positive controls can only stem from ape and monkey samples.

Based on the serological findings by Westarp et al. [1992] a clinical trial with the anti-retroviral AZT was initiated in some MND patients [Westarp et al. 1993c]. Although AZT associated mitochondrial myopathy [Dalakas et al., 1990; Mhiri et al., 1991; Chalmers et al., 1991] was not observed in the patients, this potential side effect of AZT therapy can be particularly unpleasant and possibly even harmful in MND patients. From the results presented here we conclude that there is no evidence for an FV aetiology in sporadic MND and no theoretical reason to believe that AZT therapy is of any benefit in this disease.

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